Chapter I

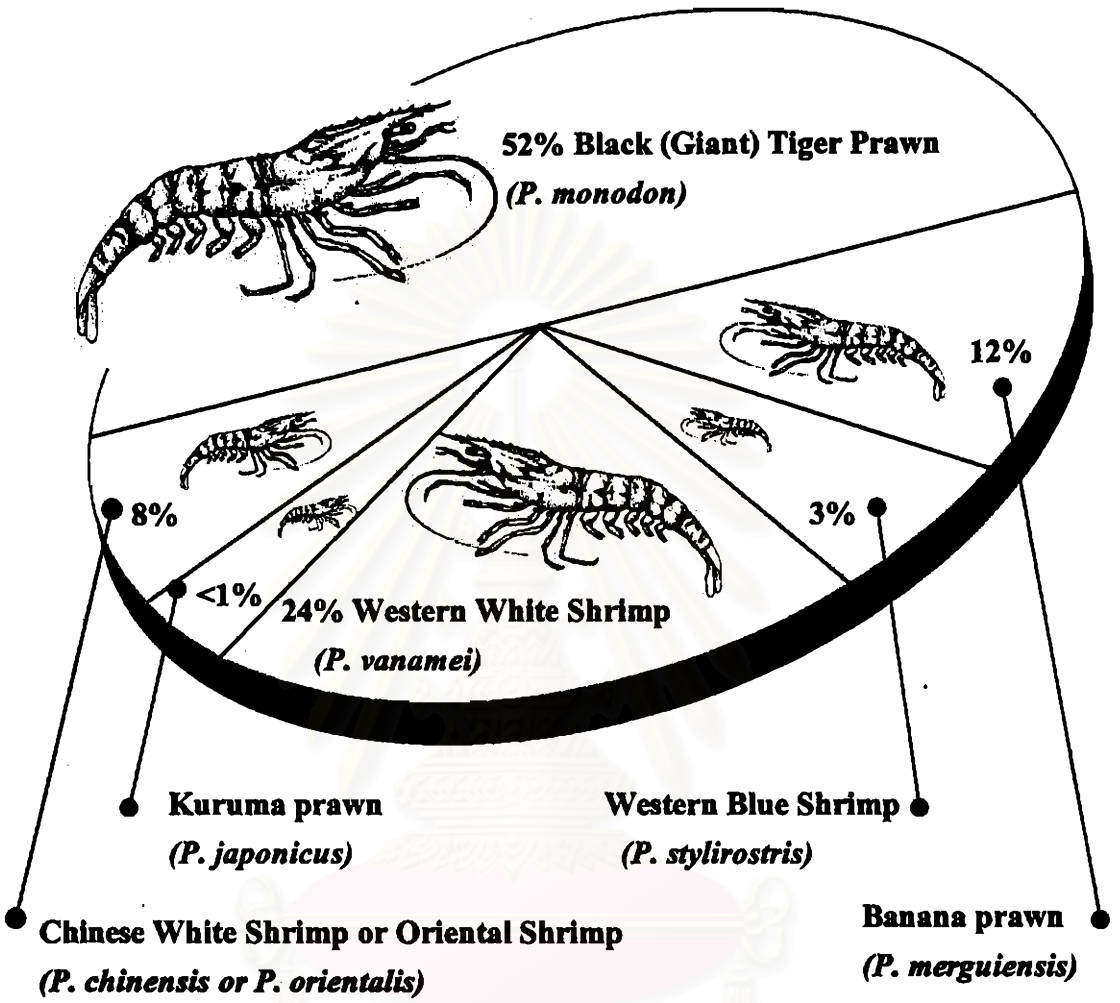
Introduction

1.1 General introduction

At present, aquaculture is the world's fastest growing food-production sector, providing an acceptable protein-rich supplement and substituting for wild aquatic animals and plants. The prawn farming is one of the common industrial aquaculture. All farm-raised and most of the wild-caught marine prawns belong to the Penaeidae. The members of this family are referred to as "penaeids". The genus name is *Penaeus*. Within this genus, the black (giant) tiger prawn (*Penaeus monodon* Fabricius, 1798) is the most important penaeid species that is caught and farmed in many tropical countries. The production of *P. monodon* was more than a half of the world's penaeid prawn production in 1998 (Figure 1.1)

In 1998, the world's prawn farmers produced an estimated 737,200 metric tons of penaeid prawns (a record-up to 12% from 660,200 metric tons in 1997). Farmers in the eastern's hemisphere produced 72% while those in the west produced only 28% of the world's production (Table 1.1). Most of the largest prawn producers are located in Southeast Asia. Thailand produced 39.6% of the eastern's production accounting as the largest producer of penaeid prawns in the world (Table 1.2). Fresh and frozen prawns from Thailand was exported to Asian countries, United States of America, Canada, Europian countries, Australia, New Zealand and others (Table 1.3).

Farming activity of *P. monodon* in Thailand has rapidly increased reflecting large annual production. The reasons for this are supported by several factors including the farming areas without serious disturbing from typhoon or cyclone, small variable of seawater salinity during seasons, and ideal soils and terrain for pond construction.



Source : World Shrimp Farming, 1998

Figure 1.1 Production of farmed penaeid prawns by species in 1998

Table 1.1 Statistics illustrating world prawn farming in 1998

Area of Prawn Farming	% of World Production	Heads-on Production (metric tons)	Hectares In Production	Kilograms per Hectare	Number of Hatcheries	Number of Farms
Western Hemisphere	28	207,000	226,800	913	457	2,494
Eastern Hemisphere	72	530,200	637,550	832	3,718	168,833
Totals	**100**	**737,200**	**864,350**	**853**	**4,175**	**171,327**

Source : World Shrimp Farming, 1998

Table 1.2 Statistics illustrating eastern hemisphere farming in 1998

Country	Percent of Production	Heads-on Production (metric tons)	Hectares in Production	Kilograms per Hectares	Number of Hatcheries	Number of Farms
Thailand	**39.6**	**210,000**	**70,000**	**3,000**	**1,000**	**25,000**
Others	28.3	150,000	200,000	750	2,000	10,000
India	13.2	70,000	140,000	500	150	100,000
Indonesia	9.4	50,000	200,000	250	300	30,000
Philippines	6.6	35,000	20,000	1,750	90	2,000
Malaysia	1.5	8,000	4,000	2,000	100	800
Sri Lanka	0.9	5,000	3,000	1,667	66	1,000
Australia	0.4	2,200	550	4,000	12	33
Totals	**100**	**530,200**	**627,550**	**832**	**3,718**	**168,833**

Source : World Shrimp Farming, 1998

Table 1.3 Thailand export of fresh and frozen marine prawns (Q = ton, V = million baht)

No.	Country	Jan - Dec 1997		Jan - Dec 1998		% Change	
		Q	V	Q	V	Q	V
1	**Asia**	**74,680.00**	**24,945.22**	**73,288.00**	**25,957.01**	**-2%**	**4%**
	China	15,484.00	4,071.87	16,796.00	4,816.63	8%	18%
	Hong Kong	4,650.00	1,632.36	4,594.00	1,147.51	-1%	-30%
	Japan	27,804.00	12,277.14	28,021.00	12,723.67	1%	4%
	Korea	4,144.00	1,201.70	2,117.00	417.67	-49%	-65%
	Singapore	13,613.00	3,452.87	15,188.00	4,793.00	12%	39%
	Taiwan	8,985.00	2,309.28	6,572.00	2,058.53	-52%	-11%
2	**U.S.A.**	**37,991.00**	**14,516.20**	**52,541.00**	**20,559.50**	**38%**	**42%**
3	**Canada**	**3,777.00**	**1,453.81**	**4,785.00**	**2,087.25**	**27%**	**44%**
4	**EU**	**13,161.00**	**3,787.82**	**15,519.00**	**5,818.82**	**18%**	**54%**
	Belgium	502.00	172.98	882.00	337.45	76%	95%
	Denmark	168.00	51.39	270.00	115.92	61%	126%
	France	4,215.00	1,197.17	3,889.00	1,477.33	-8%	23%
	Germany	1,579.00	596.41	2,404.00	1,148.27	52%	93%
	Italy	1,897.00	332.64	2,244.00	554.35	18%	67%
	Netherlands	1,253.00	411.99	1,678.00	714.37	34%	73%
	Spain	959.00	201.25	842.00	218.67	-12%	9%
	Utd Kingdom	2,588.00	823.99	3,310.00	1,252.46	28%	52%
5	**Australia**	**4,880.00**	**1,726.33**	**5,641.00**	**2,479.09**	**16%**	**44%**
6	**New Zealand**	**366.00**	**98.87**	**492.00**	**163.37**	**34%**	**65%**
7	**Others**	**2,225.00**	**655.61**	**3,910.00**	**1,278.28**	**76%**	**95%**
	Total	**137,080.00**	**47,183.86**	**156,176.00**	**58,343.32**	**14%**	**24%**

Source : Department of Business Economics

1.2 Taxonomy of *P. monodon*

The taxonomic definition of the black tiger prawn, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Scientific name : *Penaeus monodon* Fabricius, 1798

English common name : black tiger prawn or giant tiger shrimp

1.3 Morphology

Externally, the prawn can be divided into thorax and abdomen (Figure1.2). The thorax is covered by single immobile carapace protects internal organs and supports muscle origins. The eyestalks and eyes, the sensory antennules and the antennae (all paired) arise rostrally. The walking legs (pereiopods) are the thoracic appendages. Gills are formed from sac-like outgrowths of the base of the walking legs and sit in branchial chambers on either side of the thorax. The carapace extends laterally to cover

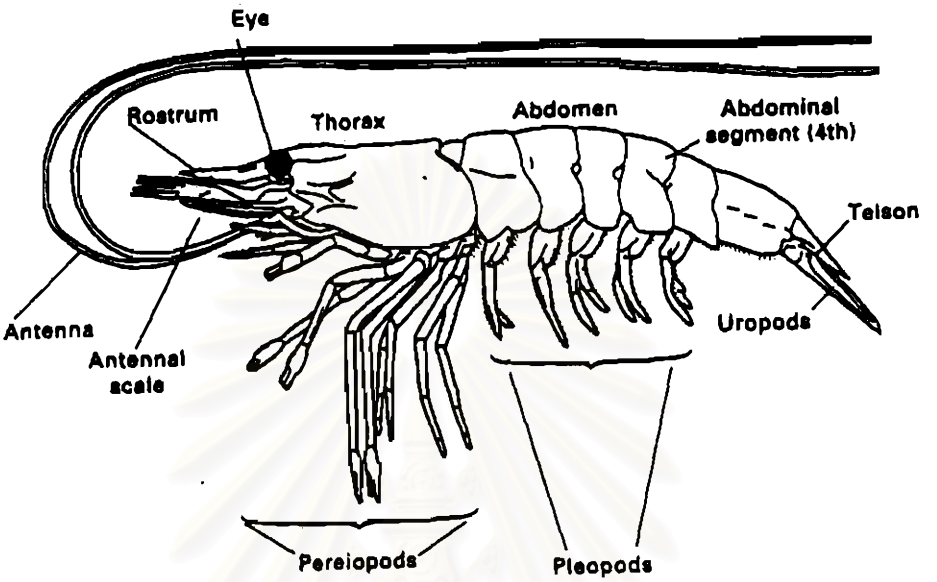


Figure 1.2 Lateral view showing important parts of *P.monodon*. (Anderson, 1993)

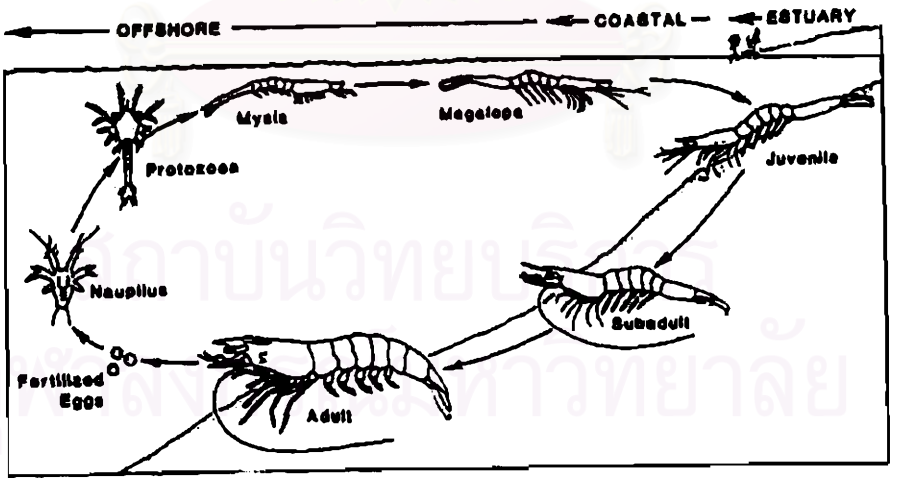


Figure 1.3 Developmental stages of the black tiger prawn, *P. monodon*, in different habitats (Bailey-Brock and Moss, 1992)

the gills completely. The abdomen has segmentation commonly observed in invertebrates. It consists of swimming legs (pleopods), which arise from each of six abdominal segments, and a tail. The tail fans comprises a telson, which bears the anus, and two uropods attach to the last abdominal segment. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawns (Anderson, 1993).

Prawns grow by periodically releasing their cuticle secreted by the epidermal cell layer, consisting of chitin and proteins. Molting starts when the epidermis detaches from the cuticle layer and begins to secrete a new cuticle. The new cuticle is soft and is stretched to accommodate the increased size of the prawn immediately after molting. In *P. monodon*, mating occurs just after the female molts. At this time the male can insert the spermatopore through the soft cuticle of the thelycum (an organ for internal storage of spermatophores). Prawns spawn directly into the sea water, and the eggs are fertilised by the stored spermatozoa at the moment of spawning (Anderson, 1993).

A live black tiger prawn has the following characteristic coloration: carapace and abdomen are transversely banded with red and white, the antennae are greyish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackish water or when cultured in ponds, the color changes to dark and, often, to blackish brown (Motoh, 1981: cited in Solis, 1988).

1.4 Life cycle

The penaeid life cycle includes several distinct stages found in a variety of habitats. Juveniles often prefer brackish waters of estuaries and coastal wetlands, while adults are usually found offshore at higher salinities and greater depths. Larval stages inhabit surface water offshore, with an on-shore migration as they develop (Figure 1.3).

Development of penaeid prawns begins with a larvae hatching from the fertilized egg to the first stage, nauplius, which occurs about 12 hours later. The larval stages consist of three to six nauplii, three protozoea and two or three mysis substages depending on prawn species. This larval development period varies with temperature and feeding levels but is usually 10-14 days. Mysis II larvae molt to become post larvae (PL) with have all appendages and organs seen in adults. Larvae exhibit planktonic behavior with antennal propulsion for swimming in nauplii, antennal and thoracic propulsion in mysis, and abdominal propulsion in megalopa. Nauplii utilize yolk granules within their body while the feeding starts in protozoea and mysis. At the mysis stage, larvae have five pairs of functioning pereiopods. The carapace now covers all of the thoracic segments. The mysis swims like adults. After this stage, larvae metamorphose to the post-larvae with a full complement of functioning appendages. The post-larvae continue to be molting as they grow. They migrate shoreward and settle in nursery areas closed to shore or estuaries, before develop to juvenile and sub-adults, which more tolerate to variety of environmental factors. Sub-adults migrate back to the sea where they finally mature and have the first corpulation and spawn. The life span of penaeid prawn is approximately 2 years (Anderson, 1993; Solis, 1988).

1.5 Distributions

The black tiger prawn (*P. monodon*) is principally distributed in the major part of the Indo-West Pacific regions. It is commonly found in the East and Southeast Africa, through the Red Sea and Arabian Gulf, around the Indian subcontinent, and throughout the Malasian Archipelagoes to Northern Australia and Japan (Figure 1.4). It is a marine species inhabits mud or sandbank bottoms at all depths from shallows to 110 meters (360 feet), so it can be caught from offshore or inshore as well as from tidal

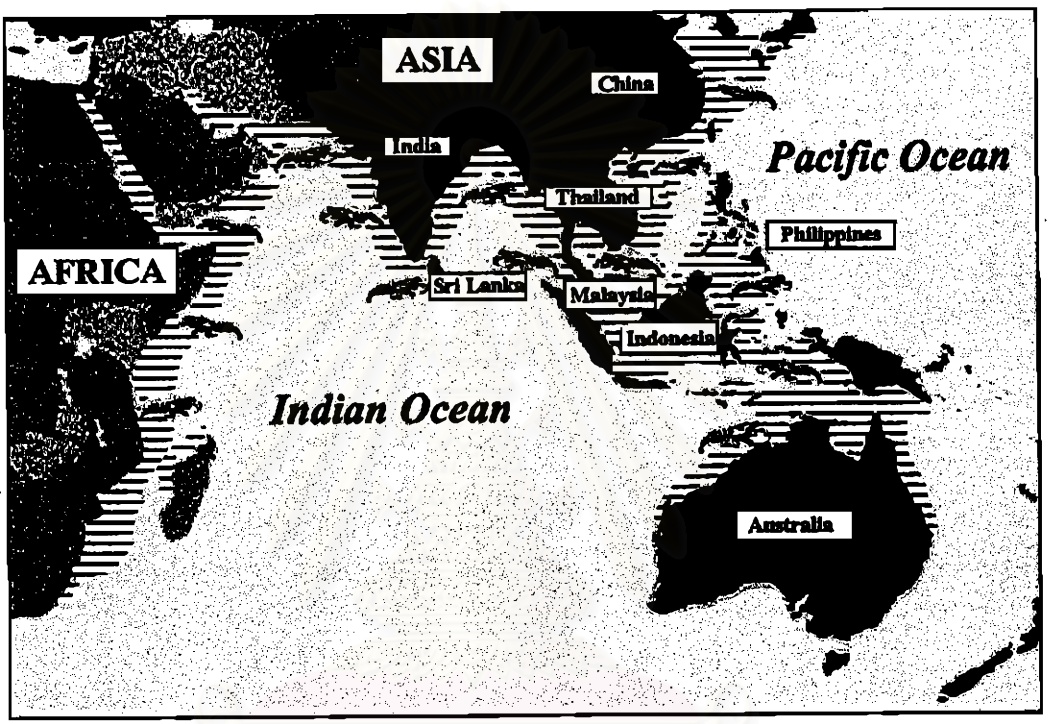


Figure 1.4 Geographic distributions of *P. monodon* in the Indo-West Pacific regions (Grey et al., 1983)

zones (or ponds). This species is one of the most important cultivated prawn species in Asia (Dore and Frimodt, 1987).

1.6 Exploitation

Due mainly to the strong demand and the high price of *P. monodon* in international markets, *P. monodon* industry in Thailand has rapidly expanded. This causes the increasing use of wild female broodstock, because farming of *P. monodon* relies entirely on wild females for the seed production (Figure 1.5). To minimize environmental impacts and to fully realize the value of genetic diversity of natural populations, prawn aquaculture must break its reliance on wild postlarvae. As in farming of other agricultural species, domestication is a better alternative which allows convenient disease prevention and control. The genetic selection may also be achieved through this process. As a result, domesticated *P. monodon* should be substituted for the use of wild females as the only source of postlarvae production in the prawn industry.

In prawn farming, genetic selection for fast-growing and disease-resistant traits would be most desirable, as both would decrease the risk of a loss of the production. The use of a small number of breeders for production of the stocks in hatcheries and those will be released to the natural environment, is the primary cause of a loss of genetic variation and for the incidence of inbreeding of *P. monodon*. Thus, knowledge on the sizes of *P. monodon* broodstock effectively contributing to the next generation and estimation of the inbreeding coefficient is of particular importance (Perez-Enriques, Takagi, and Taniguchi, 1999).

Genetic markers that demonstrate genetic relationships of pedigree of communally reared aquaculture populations, would allow high-intensity selection programs to take place in penaeid farms production. DNA fingerprints or profiling

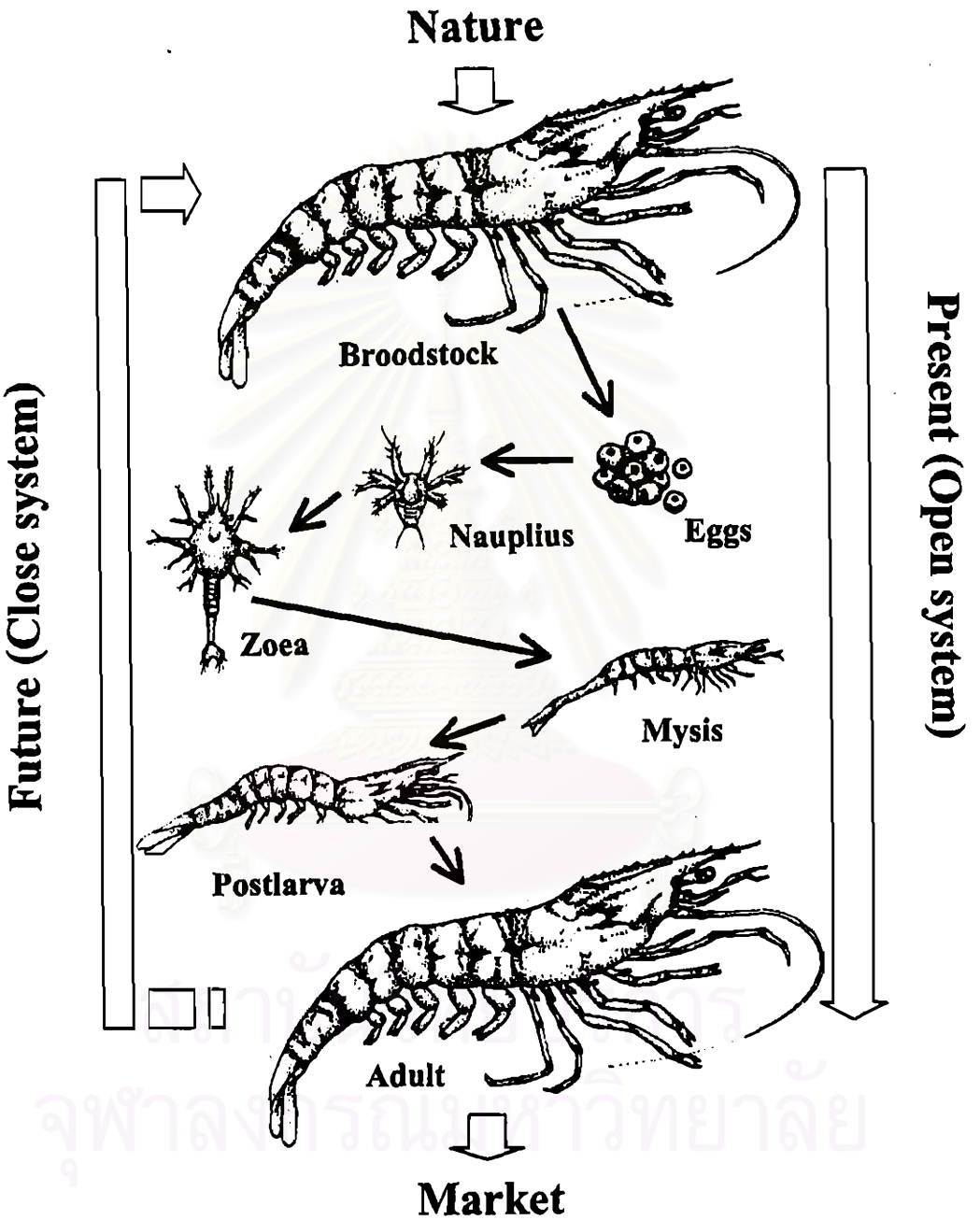


Figure 1.5 Production systems of *P. monodon*

markers such as those from microsatellites probably the most appropriate genetic marker for selection programs because there are generally inherited in a Mendelian way and are thus potentially useful for pedigree tracing.

1.7 Molecular genetic markers

1.7.1 Protein markers

Allozyme analysis is the most using protein-based approach for population genetic studies in various species. An allozyme refer to that produced by different alleles at the same chromosomal DNA locus (Park and Moran, 1994). The polymorphic allozymes can be detected by electrophoresis based on the property that proteins with different net charges migrate at different rates through a gel matrix when exposed to an electric field (Avise, 1994). The charge characteristics of proteins vary with pH of the running buffer reflecting their movements towards the positive or negative poles (Morizot and Schmidt, 1990) Therefore, proteins are electrophoretically separated according to their net charges, sizes and shapes (Cooper, 1977). The advantages of allozyme analysis are that the technique is convenient and cost-effective. Large numbers of specimens can be analyzed within a limitation of time. Moreover, several allozyme loci can be simultaneously examined. Accordingly, allozyme analysis is a technique of choice to begin with when the species under investigation has not been reported for any molecular data. The systems are generally useful across various taxonomic levels (Memzies, 1981; Mitton and Koehn, 1985; Todd and Hatcher, 1993; Ward and Elliott, 1993 all cited in Park and Moran, 1994). Allozyme markers are transmitted in a co-dominant manner. Unfortunately, this technique possesses some technical limitations. Only histochemical stains available for some enzymes can be carried out. The most serious problems to apply this approach to a practical selective breeding program are limited by the levels of detected polymorphism and the

broodstock, in most cases, need to be sacrificed for the analysis. Isoloci can cause difficulties resulted from co-migration of bands originating from different loci (Buth, 1990).

1.7.2 DNA markers

Domestication of *P. monodon* through selection programs needs molecular genetic markers that can trace parent-offspring links. These genetic markers should have sufficient variation that can distinguish one individual from another with high probability. Highly variable regions of DNA such as variation number of tandem repeats (VNTR) are mostly used to demonstrate more fine scale of genetic variation than that of protein markers. VNTR markers are characterized by a core sequence consisting of a number of identical repeated sequences. They can be divided into three categories; satellite, minisatellite, and microsatellite, based on the repeated length. (O'Reilly and Wright, 1995)

1.7.2.1 Satellites

Satellite DNA was initially termed for DNA consisting of long repeat units (hundreds to thousands of base pairs) which was first identified among all VNTR. Due to the abundance of repeats and their slightly different base composition compared to bulk genomic DNA, this class formed a separate, 'satellite' band which is distinguishable from the main genomic band in the equilibrium density centrifugation. In some mammals, certain satellite DNA may occur in millions of copies per genome (Alberts et al., 1983). However, they are not as variable in size as the other members of highly repetitive DNA family.

1.7.2.2 Minisatellites

Minisatellite DNA was termed from reduced length of repeat units, compared to satellite DNA. Minisatellites is a repeating DNA sequence ranging between 15-70 bp per unit and 0.5-30 kb in cluster size (Koreth et al., 1996). Minisatellites are found within non-coding regions of genomic DNA. Increases and decreases in the lengths of these result from changes in the number of repeat copies residing within the region and, hence, it is called variable numbers of tandem repeats (Avise, 1994). The mechanisms generating variability in minisatellites are still inconclusive. Several models have been suggested including unequal crossing over between homologous chromosomes during meiosis, replication slippage, and gene conversion (Wolff et al., 1989). Differences in length of minisatellites can be not only from the number of copies of repeats, but also from interspersion patterns of different types of repeats for which comprise the array. The variation of minisatellites can be conventionally detected due to difference in length between restriction sites. The determined DNA patterns are called DNA fingerprints, which was first reported by Jeffreys et al. (1985). The DNA profiles used for individual and parental analysis can be from either multi- or single locus of VNTR loci.

In multilocus DNA fingerprinting, length variation is simultaneously surveyed at many VNTR loci. Due to the large number of loci examined and the extremely variable nature of this particular class of repeated DNA, each profile of bands is usually highly informative and individual-specific. Practically, conventional multilocus DNA fingerprinting was not appropriate to use for large numbers of specimens because protocols often do not generate reproducible results, less sensitive (requiring more DNA) and more time consuming methodology (Southern blot and hybridization) than other PCR-based approaches.

In single locus profiling, allellic variation is surveyed at individual VNTR loci, using conventional or PCR-based methods. The first approach involves restriction endonuclease digestion of genomic DNA, Southern blotting and probing with labelled DNA recognizing a single VNTR locus, preferably the unique flanking region of a particular locus. The second method is PCR amplification of the locus using primers flanking the array. Jeffreys et al. (1991) was the first who developed a more expedient PCR-based method from the sequence variation within single molecules, termed minisatellite variant repeat-PCR (MVR-PCR also referred as digital DNA fingerprinting). This resulted in the rapid attenuation of signal strength of bands representing increasingly distal repeat positions.

The single locus DNA fingerprinting obviate several major problems associated with the multilocus method. First, band profiles are much less complicated, and co-migration of alleles from non-homologous loci is unlikely to occur. Second, since bands can be designed to a given locus, allele frequencies can be estimated. Third, with the inclusion of proper allelic standards, comparisons are possible across different gels. Although, this DNA fingerprinting gave a good result, PCR products may not be properly amplified because of the large sizes. The non-amplifying alleles may interfere unambiguous conclusions of the results.

1.7.2.3 Microsatellites

Microsatellites, also called simple sequence repeats (SSR) or short tandem repeats (STR) DNA, are tandem arrays containing short nucleotide motifs of 1-6 nucleotide repeats for approximately 10-50 copies (Hearne et al., 1992). Microsatellite are highly abundant and randomly dispersed in most eukaryote genomes (Valdes et al., 1993; Weissenbach et al., 1992; Wright, 1993). It was estimated that one microsatellite locus may be found every 10 kb in eukaryotic genomes (Tautz, 1989). Due mainly to

high mutation rate of microsatellite loci ($1 \times 10^{-5} - 5 \times 10^{-4}$ per generation), they exhibit high allelic variation and heterozygosity levels (Hearne et al., 1992; Wright and Bentzen, 1994). Like minisatellite, variability in microsatellites regions arise from changing in the number of repeated sequences which is proposed to be from slipped-strand misparing or slippage during DNA replication (Schlotter and Taulz, 1992; Moxon and Wills, 1999).

Tautz et al. (1989) was the first to demonstrate microsatellite polymorphism by PCR amplification using primers complementary to unique flanking domains. Some microsatellite loci are highly polymorphic and allelic-variants at these loci are inherited in a Mendelian fashion. Because of their advantages (abundance, distribution, polymorphism, easy-isolation and assay by PCR from minute amounts of tissue), microsatellites are considered a general source of genetic markers for DNA fingerprinting. As a result, microsatellites are potential for several applications particularly when homozygotes are needed to dissociate from heterozygotes.

Many applications of microsatellite markers in aquaculture have been described. Microsatellite markers have been used to study paternal and maternal effects on offspring growth and survival in communally reared rainbow trout, *Oncorhynchus mykiss* (Herbinger et al., 1995), and used for breeding programmes in the sea bass, *Dicentrarchus labrax* (Garcia de Leon et al., 1998). Perez-Enriquez et al. (1999) analyzed genetic variability and pedigree tracing of a hatchery-reared stock of red sea bream (*Pagrus major*) used for stock enhancement. Herbinger et al. (1999) determined the early growth performance of Atlantic salmon full-sib families reared in single family tanks versus that in mixed family tanks. Volckaert et al. (1999) demonstrated survival, growth and selection in a communally tank of multifactorial crosses of African catfish, *Clarias gariepinus*.

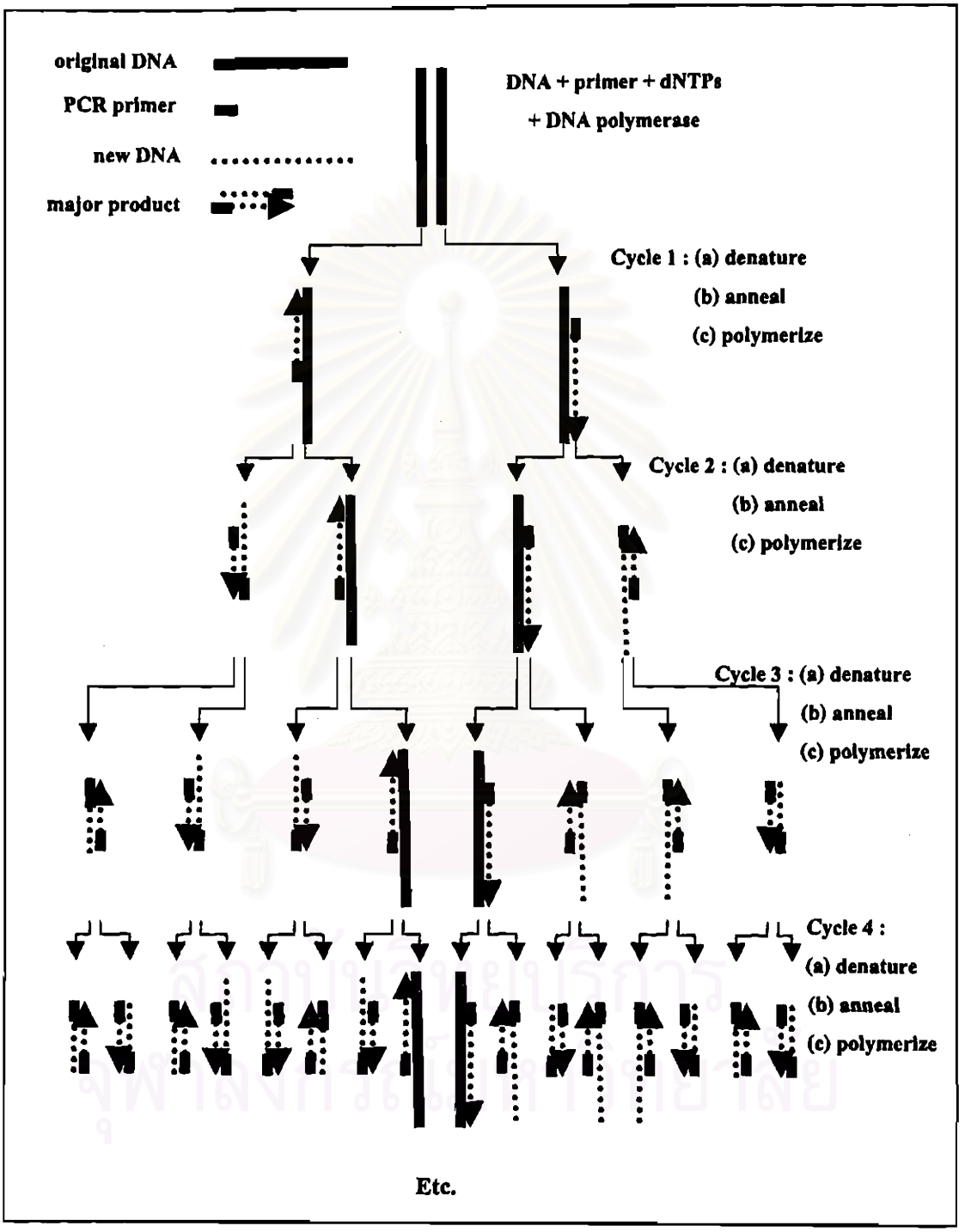


Figure 1.6 The schematic diagram of polymerase chain reaction (PCR)

Allelic variations of a particular microsatellite locus are detected through polymerase chain reaction (PCR). The theoretical basis of PCR is outlined in Figure 1.6. After amplification, the products are fractionated for their length polymorphism using agarose (usually for tetranucleotide microsatellites) or polyacrylamide gels (di- or trimeric microsatellites) with either non-radioactive or radioactive methods. Generally, detection of amplified microsatellites by autoradiography (labeled 5′end of one of the primers, electrophoresed of the products and exposed the gel with the X-ray films) are more sensitive and commonly used. Radioactive detection of microsatellite gives cleaner results but less cost-effective than does the non-radioactive approach.

Non-radioactive detections are compared of ethidium bromide staining, silver staining and fluorgraphy. Staining of the electrophoretic gels with ethidium bromide is the simplest visualization approach but the lowest sensitivity compared with the remaining techniques. At least 10 ng of double stranded DNA fragments are required for detection (Bethwaite et al., 1995). Silver staining offers better sensitivity over ethidium bromide staining (pg quantities of DNA) and has been widely used for qualitative assessment of microsatellite allelic bands (Love, 1990). However, silver stain produces high and variable background caused by non-linear deposition of the silver. Detection of microsatellites using fluorescence dyes in coupling with the automated DNA sequencer yield significantly more rapid and reliable results. This technique is suitable for detection of multiplex amplification of multiple microsatellite loci. Nevertheless, the use of this system is limited by the cost and the availability of the automated sequencer.

Allelic ladder is a standard size marker consisting of all or most of the known alleles for a particular locus. This marker can be constructed for selected systems to add precision and accuracy to identify of alleles at each locus. Applications of allelic ladder are rapid and are direct comparisons of suspected alleles with the allelic ladder

components rather than by measurement and calculation of target fragment sizes. This method is also more accurate than comparison with classical size standards such as bacteriophage or plasmid markers. These tools can also be applied in development of high-throughput analytical methods. For example, multiple samples amplified at a single locus can be amplified and loaded at different times with allelic ladders. Likewise, the same sample may be amplified separately at several loci and the amplified samples mixed prior to gel electrophoresis. This mixture can be compared with that of allelic ladders for the same loci. Interpretation is straightforward as long as the allele size ranges of mixed loci do not overlap.

Multiplex analysis of microsatellite loci helps to minimize labor, materials, and analysis time. One approach to reaching this goal involves mixing samples from several individual amplifications prior to loading them on the gel. The other approach such as multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 by Chamberlain (Chamberlain et al., 1988: cited in Henegariu et al., 1997) this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphism, or quantitative assays and reverse transcription PCR. To construct multiplex sets, loci having compatible amplification protocols and different allele size ranges were simultaneously amplified in one reaction tube, separated by denaturing polyacrylamide gel electrophoresis and detected by silver staining or autoradiography. To correctly analyze all loci in the multiplex, it is desirable to have similar amplification yields for different loci. Thus, multiplex sets were designed to permit amplification of each locus simultaneously without generating new PCR artifacts.

1.8 Genetic markers in penaeid prawns

In penaeid prawns, molecular genetic markers have been developed for comprehensive population studies and breeding program. Tassanakajon et al. (1997) reported that the primer UBC 428 generated a RAPD marker that was found only in *P. monodon* originating from Satun-Trang, located in the Andaman Sea. This suggested the potential use of this marker as a population-specific marker in this species. Microsatellite markers were isolated and applied for analyzing genetic diversity in the western white prawn, *P. vannamai* breeding programs (Wolfus et al., 1997). Isolation and characterization of microsatellite sequences from *P. monodon* were recently described by Tassanakajon et al. (1998). Results from screening of the genomic library indicated that $(GT)_n$ microsatellites are more abundant than $(CT)_n$ in *P. monodon* genome. Two microsatellite loci, CUPmo18 and CUPmo386, were successfully isolated. The abundance and sequence types of tri- and tetranucleotide microsatellites in *P. monodon* have been reported (Pongsomboon et al., 2000). In 79 positive clones isolated, the $(GATA)_n$ repeat was found at the highest frequency, followed by the $(GAA)_n$ while the other sequences were rare or not found. Xu et al. (1999) reported the abundance and informative nature of *P. monodon* microsatellites and their potential for cross-species amplification making them useful for genetic studies of penaeid shrimp species. Moore et al. (1999) developed and applied microsatellite and amplified fragment length polymorphism (AFLP) markers for establishing pedigrees, linkage mapping and identifying quantitative trait loci (QTL) influencing commercially important traits in *P. japonicus*.

1.9 Objective of the thesis

The aim of this study to develop a simple and reliable method for DNA typing of *P. monodon* using the microsatellite technique. Eight microstellite loci namely

CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6, CSCUPmo7, CSCUPmo9 and CSCUPmo11, developed by Pongsomboon et al. (2000), were tested to determine the level of polymophism and microsatellite allelic patterns by isotopic detection method. Several steps of DNA typing were simplified. These included DNA isolation, optimization of each amplified microsatellite locus for non-isotopic detection methods, appropriate size-separation and visualization of microsatellite allelic bands for each microsatellite locus. Allelic ladders of each microsatellite locus and multiplex analysis were developed to increase efficiency of detection. The improved methods should be fast, simple and cost-effective for use with a large number of samples in breeding programs and population genetic studies of *P. monodon*.